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ERYTHROCYTES IN CYTOPLASM AND NUCLEI OF LIVER CELLS.

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THE present note is concerned with the occurrence of erythrocytes in the cytoplasm and nuclei of the liver cells of rats treated with allylformate and with urethane. These observations were made in the course of our studies on the influence of various capillary poisons on the liver, the results of which will be reported elsewhere (Doljanski and Rosin, 1944).

MATERIAL AND METHODS.

Twenty albino rats weighing 80–100 g. were used in these experiments. Of these, eight rats were treated with urethane, the remaining twelve with allylformate. Both urethane and allylformate were given intraperitoneally for 4–14 days. The initial dose of urethane was 0·5 c.c. of a 10 per cent. solution; this was increased daily by 0·1 c.c. until the death of the animal occurred. The initial dose of allylformate was 3 mg. dissolved in 2 c.c. of Ringer's solution; this was increased daily, or on every second day, by 1 mg. until the death of the animal.

Pieces of liver were fixed in Zenker's fluid and embedded in celloidin-paraffin according to Péterfi. Sections of five microns in thickness were stained with haematoxylin-eosin and with Giemsa's stain. Lepehne benzidine and prussian blue tests were applied for sections of suitably fixed material.

OBSERVATIONS.

Damage of the portal and sublobular veins and of the endothelial lining of the sinusoids is a most constant feature in the liver of animals treated with urethane or allylformate. The disorganization of sinusoidal endothelium as well as the degeneration and rupture of the wall of the greater veins results in the passage of plasma from the vascular system into the parenchyma. Thus the pericapillary spaces become greatly distended, and the portal and sublobular veins become surrounded by areas of extravasated fluid. With further progress the damage of the vascular wall becomes more pronounced, and there is extravasation of erythrocytes into the pericapillary spaces and more or less extensive haemorrhage, mainly around the portal tracts. The alterations of the vascular system are of the same type in both urethane and allylformate poisoning, but are more pronounced in the latter. The intensity of the parenchymatous damages varies with the poison used. In allylformate poisoning the

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liver cells in the peripheral zone of the lobule sustain severe injuries, which rapidly lead to necrosis ; in urethane poisoning the injuries of the liver cells are only slight.

In and around the haemorrhagic areas the extravasated erythrocytes are found in direct contact with the liver cells. A careful examination of the preparations reveals the presence of red blood cells, not only in the immediate surroundings of the liver-cells, but even within their cytoplasm. Here they can be encountered singly or in groups ; in the latter case they form larger, spherical aggregations. At first the agglomerated erythrocytes present a completely unchanged appearance, but subsequently they become fused into a single round or oval body, in which the individual corpuscles may still be recognized for a time ; later the individual erythrocytes lose their identity and condense into massive, sharply outlined balls, 7–25 microns in diameter. These spherical inclusions sometimes present a hyaline appearance ; sometimes they are slightly spongeous or delicately granular in structure. At first the erythrocytes lying in the liver-cell cytoplasm, as well as the spherical bodies originating from them, stain deep pink with haematoxylin-eosin and pink by Giemsa's method ; gradually the affinity of the inclusions for eosin decreases and they colour a faint pink to a pale pinkish-lilac with haematoxylin-eosin and faint yellowish-pink to greyish-violet with Giemsa's stain ; they give a negative staining reaction for fibrin (Weigert). Frequently inclusions are found which contain well-preserved red blood cells embedded in a homogeneous matrix (Figs. 1, 2, 3 and 4).

There is mostly only one inclusion body in a liver cell, but occasionally two or even three fairly large spherical inclusions are seen within the body of the individual cell. When the inclusions are voluminous they push the nucleus of the liver cell aside. The cytoplasm of erythrocyte-bearing liver cells is swollen and delicately vacuolated, but shows no other observable alterations. The spherical inclusion bodies are almost invariably situated in a vacuole.

In the case of rats which have received repeated intraperitoneal injections of allyl-formate, erythrocytes may occasionally be found, not only in the cytoplasm, but also in the nuclei of the liver cells (Fig. 5). Their number in a single nucleus varies from 1–15. They lie at first completely unaltered within the nuclei ; subsequently they fuse into a single, spherical or ovoid body, in which the outlines of the erythrocytes may be recognized for a time ; later they become indistinct and the inclusions exhibit a homogeneous, almost hyaline appearance. The nuclei which contain erythrocytes are as a rule appreciably enlarged ; their diameter becomes as much as four times the average diameter of the nuclei in the section. In rare cases, where only isolated erythrocytes are found in the nuclei, the latter show no clear evidence of injury ; the nucleoplasm presents a pale, homogeneous appearance, the basophilic chromatin of the nuclei is unaltered and lies between the inclusions and the unchanged nuclear membrane ; sometimes the intranuclear red blood corpuscles are situated in a vacuole. If the normal or distorted erythrocytes fill the entire intranuclear space, as is mostly the case, all that is left of the nucleus is the thickened nuclear membrane, which surrounds the inclusion as a heavy basophilic rim. The nuclear membrane is sharply outlined interiorly, while the outer margin frequently displays budlike protuberances. The basophilic chromatin and nucleoli may be found aligned along or intimately fused with the nuclear membrane, but in the vast majority of cases they are wholly absent. Generally the membrane of the affected nuclei is continuous, but not infrequently the membrane persists only in a sector, the rest having entirely disappeared. The intranuclear erythrocytes show the same staining properties as those situated in the cytoplasm ; the hyaline spherical bodies derived from them stain at first like the red blood cells, but their affinity to eosin gradually decreases.

The intracytoplasmatic and intranuclear erythrocytes, as well as the hyaline

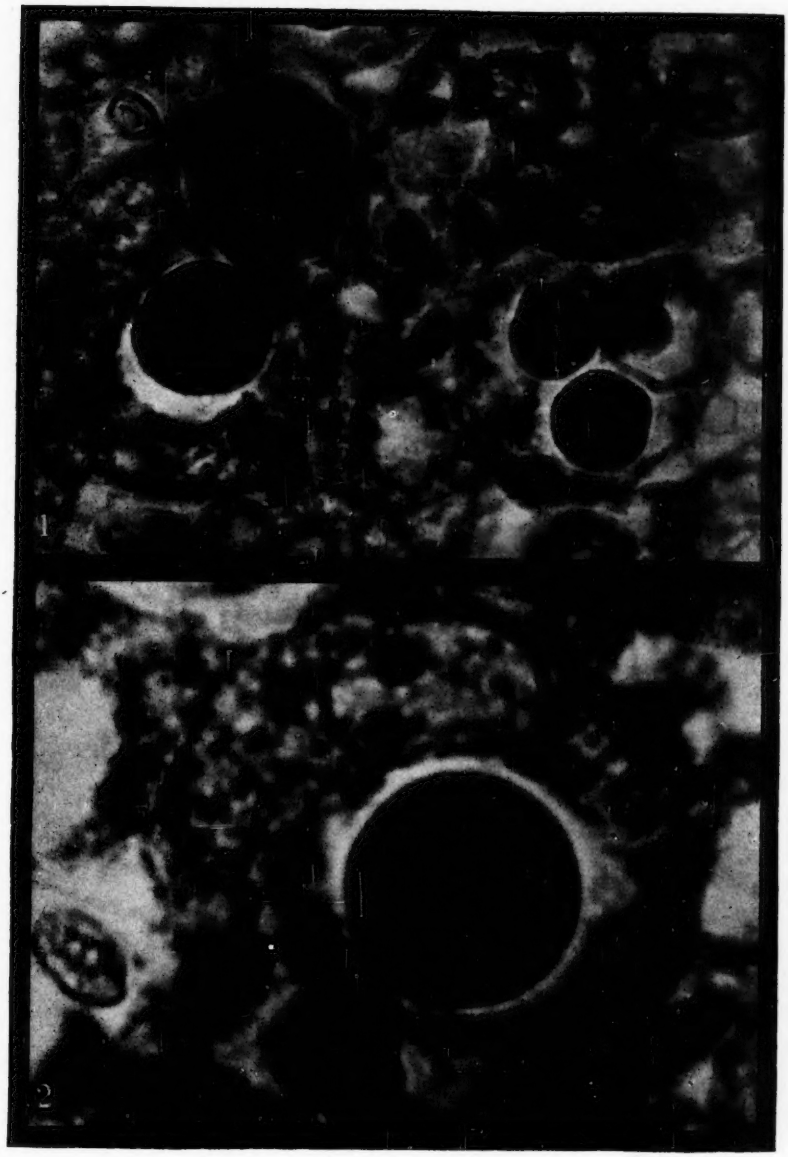

FIG. 1.—Agglomerated erythrocytes and spherical bodies in liver-cells of a rat injected with urethane. Haematoxylin and eosin stain. × 1700.
FIG. 2.—Spherical body in a liver-cell of a rat injected with urethane. × 3000.

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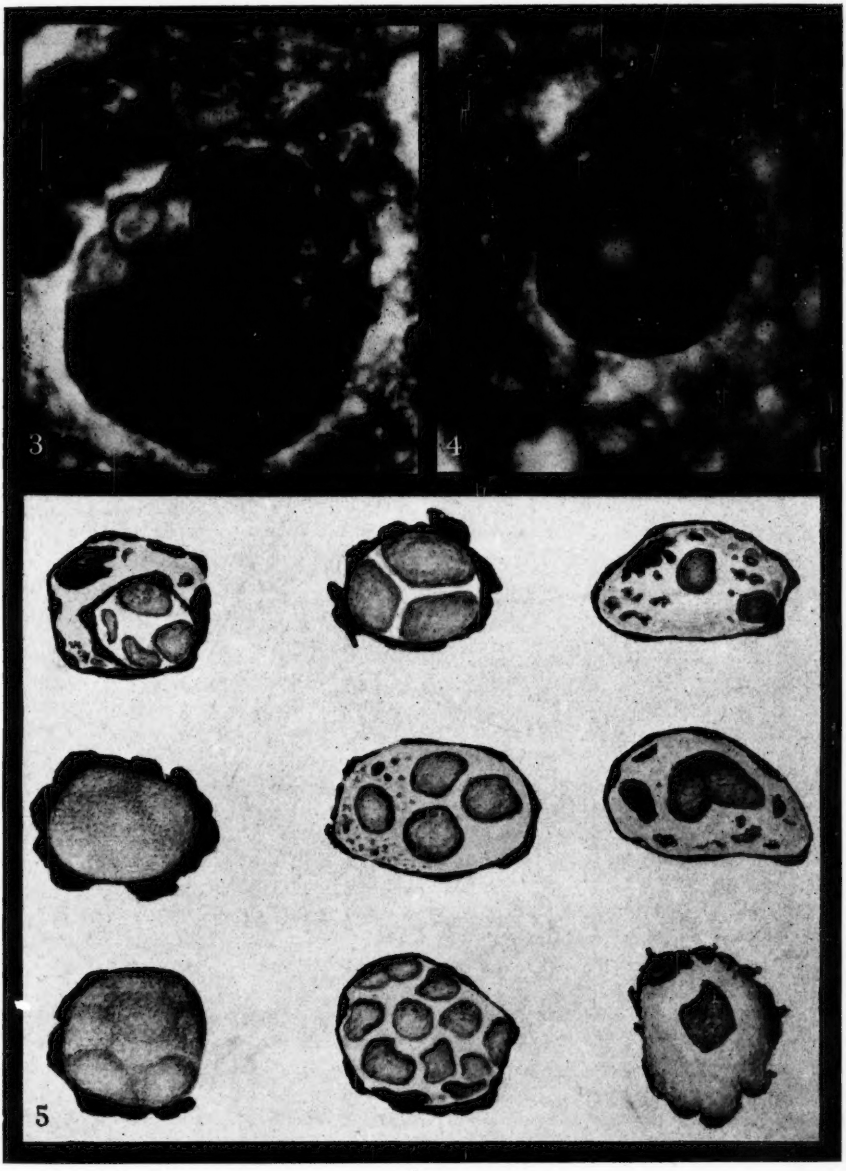

FIGS. 3 and 4.—Agglomerated erythrocytes and hyaline ball in liver-cells of rats injected with allylformate. Haematoxylin and eosin stain. × 3000.

FIG. 5.—Erythrocytes in the nuclei of hepatic parenchymal cells. Rats injected with allylformate. Fixed in Zenker's fluid; haematoxylin and eosin stain. Drawn at a magnification of 1500–2500.

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bodies derived from them, show at first a positive Lepehne benzidine test ; with further progress of the changes the brown colour disappears. In experiments of short duration, neither the cytoplasm nor the nuclei of the inclusion-bearing liver cells give a positive prussian blue reaction for iron, but in the liver of rats treated for two weeks, small deposits of iron are occasionally encountered in the cytoplasm of affected cells.

The degree of incidence of the intracytoplasmatic and intranuclear erythrocytes is variable. Of eight rats treated with urethane, three showed erythrocytes in the cytoplasm of the liver cells ; of twelve rats treated with allylformate, the intracytoplasmatic erythrocytes were found in six. In some animals the intracytoplasmatic erythrocytes are found in abundance ; in others they are present in only a small percentage of the liver cells. The intranuclear erythrocytes are of much rarer occurrence ; they were met with exclusively in allylformate rats, and of these in only four out of twelve. They were plentiful in one rat, but sparse in the remaining three animals.

DISCUSSION.

As far as we can trace, the only mention of the presence of whole erythrocytes in the nuclei of the liver cells is that of Browicz (1902) made more than 40 years ago. These findings were made in dogs, after intravenous injection of haemoglobin and defibrinated foreign blood, as well as in normal dogs " during digestion."

The presence of erythrocytes in the cytoplasm of liver cells was first reported by Vereecke (1896) in dogs, chickens, and frogs, after injections of peptone, as well as in dogs poisoned with curare, and by Schmaus and Boehm (1898) in the livers of white mice and guinea-pigs treated with phosphorus. Later Roessle (1907) observed accumulation of red blood corpuscles in liver-cells in a case of human haemochromatosis. He considered these accumulations a result of phagocytosis, following injury of both hepatic cells and erythrocytes. Heinrichsdorff (1924) carried out histological examinations on cases of human jaundice of diverse aetiology, in jaundice produced in cats by ligation of the common bile duct, and in dogs by injections of toluylene diamine, as well as in pigeons after poisoning with arsenic. In all these cases he found erythrocytes within the liver cells. This author believes that the hepatic cells become permeable to red blood corpuscles owing to a toxic agent which also causes capillary failure. Dick (1925) observed erythrocytes in the liver cells in a case of haemolytic jaundice in man. Graeff (1927) reports similar findings in the liver of guinea-pigs poisoned with amanita toxin. Weatherford (1935) described the same phenomenon in anaphylactic shock in dogs. Some of the authors believe that the ingested red corpuscles are transformed within the liver cell to bile.

The mechanism of uptake of erythrocytes by the liver cells is as yet completely obscure. Our observations, as well as those of the earlier authors, are insufficient to explain how the accumulation of erythrocytes in the liver cells occurs. *Prima facie* there are two possibilities: (1) the liver cells may become actively phagocytic, or (2) the blood may be forced into the parenchyma cells as a result of increased pericapillary pressure following injury to the capillaries ; in consideration of the observations of one of us (Doljanski, 1931) in liver cell cultures where, in spite of especially favourable conditions, the parenchyma cells were never observed to carry on phagocytosis, we are inclined to accept the latter interpretation as the more probable. At any rate, the fact that the erythrocytes are found not only in the cytoplasm, but also in the nuclei of the liver cells, indicates that the process at work is not one of common phagocytosis.

The origin of the spherical inclusion bodies found in the cytoplasm of the liver cells is obvious. They arise by coalescence of ingested erythrocytes, a process which

may be followed step by step. The occurrence of homogeneous spherical inclusion bodies in the cytoplasm of the hepatic cells has been observed by Vereecke (1896), whose interpretation of their origin is similar to ours. The possibility that other blood ingredients, which may be taken up by the hepatic cells simultaneously with the erythrocytes, may be implicated in the formation of these bodies should also be considered.

It is to be noted that the ingested erythrocytes and the products of their disintegration are always different from intranuclear crystals, which were described in liver cells by Grandis (1889), Browicz (1898, 1902), Brandts (1909), Berg (1929), Weatherford (1938), and others.

There is little doubt that the inclusion-laden hepatic cells, unless they are highly damaged, have the power of breaking down the ingested red cells. Their disintegration is primarily manifested in loss of haemoglobin, which is followed by a gradual disintegration of the ingested material. During the period covered by our observations there was no appreciable deposit of iron, nor of bile pigments, in the hepatic cells.

It appeared important to us to draw attention to the findings reported above, since the presence of erythrocytes in the nuclei and cytoplasm of liver cells in certain pathological conditions does not seem to have received due consideration in modern literature. This peculiar phenomenon is probably not rare and deserves a thorough attention. The mention of this phenomenon seems justified for yet another reason. Eosinophilic inclusions in the nuclei and in the cytoplasm of the liver cells have been repeatedly described in cases of poisonings (Blackmann, 1936), in anaphylactic conditions, or after injection of different proteins (Weld, von Glahn, and Mitchell, 1941), in various diseases associated with necrotic and haemorrhagic lesions of the liver parenchyma (Cowdry and Scott, 1930; Hass, 1935; Belt, 1939), and in different animals and man independent of any established cause (Goodpasture, 1918; Findlay, 1932; Russell, 1932; Nicolau and Kopciowska, 1936; Pappenheimer and Hawthorne, 1936; Campbell, 1939; Andrew, Brown, and Johnson, 1943). The significance of these inclusions and their possible connection with an actual or previous virus infection has been much debated in current literature and is still obscure. It seemed to us that in any attempt to explain the significance of these changes in the cytoplasm and nucleus of liver cells, which have been described under the name of "inclusion bodies" and which appear in conditions unassociated with known viruses, the fact should be borne in mind that red blood corpuscles and their disintegration products are perhaps not of infrequent occurrence in the cytoplasm and the nuclei of altered liver cells. The occasional similarity between intracellularly located distorted red blood cells and structures variously described as "inclusion bodies" can hardly be denied. This resemblance may doubtlessly lead to confusion.

SUMMARY.

The presence of erythrocytes in the cytoplasm and nuclei of liver cells in rats treated with urethane or allylformate is reported and the significance of these findings is discussed.

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INFLUENCE OF MILK FACTOR ON THE INCIDENCE OF BREAST CANCER INDUCED BY OESTRONE.

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IN a previous paper (Dmochowski and Gye, 1943) the influence of the milk factor on the development of breast cancer in certain inbred strains of mice was described. The results obtained confirmed the findings of Bittner (1940*a*) and other workers, and demonstrated the general validity of the role played by the milk factor in the origin of spontaneous breast cancer in inbred strains of mice.

Lacassagne and Danysz (1939) and later Twombly (1940), Bittner (1940*b*, 1941), and Shimkin and Andervont (1941) found that foster nursing of low cancer strains of mice by high cancer strain females increases the incidence of mammary cancer in the low cancer strain mice which are treated with oestrogenic hormones.

The experiments described in this paper were undertaken for the purpose of determining whether these findings apply to pure line strains of mice which have been bred in the laboratories of the Imperial Cancer Research Fund.

EXPERIMENTAL PART.

The experiment was started in October 1941 by brother to sister matings of mice belonging to " S " low cancer and R III high cancer strains. Pregnant mice were examined twice daily for litters, and as soon as they were born half of each " S " strain litter was transferred to a mother of R III strain, and at the same time half of the litter of R III strain was given to the " S " strain mother.

In this way litter mates of strain R III mice were obtained, of which half were nursed by their own mothers and half were foster nursed by females of " S " strain,

Table I.

Sex.	"S" suckled by "S." Not painted. Number of mice.	Number of tumours.	"S" suckled by "S." Painted. Number of mice.	Number of tumours.	"S" suckled by R III. Not painted. Number of mice.	Number of tumours.	"S" suckled by R III. Painted. Number of mice.	Number of tumours.	R III suckled by R III. Not painted. Number of mice.	Number of tumours.	R III suckled by R III. Painted. Number of mice.	Number of tumours.	R III suckled by "S." Not painted. Number of mice.	Number of tumours.	R III suckled by "S." Painted. Number of mice.	Number of tumours.
Males	14	0	19	1* (5·2%)	14	0	31	12 (38·7%)	16	0	14	8 (57·1%)	16	0	16	1 (6·2%)
Females	13	0	21	1 (4·7%)	16	4 (25%)	21	11 (52·3%)	16	13 (81·2%)	13	9 (69·6)	19	1 (5·2%)	17	1 (5·8%)

* In another series of experiments more than 120 male mice of the "S" strain were treated with oestrone for 12 months and not a single tumour was induced.

Table II.

Strain of mice.	Suckled by—	Sex.	Painting with oestrone.	Number of mice.	Breast tumours in mice in months after administration of oestrone. 4. No.	5. No.	6. No.	7. No.	8. No.	9. No.	10. No.	11. No.	12. No.	13. No.	14. No.	15. No.	Number of tumours.	Average tumour age (months).
"S"	"S"	M.	—	14	—	—	—	—	—	—	—	—	—	—	—	—	—	—
"S"	R III	M.	—	14	—	—	—	—	—	—	—	—	—	—	—	—	—	—
"S"	"S"	M.	+	19	—	—	—	—	1	—	—	—	—	—	—	—	1	8
"S"	R III	M.	+	31	—	1	1	1	—	1	2	1	4	1	—	—	12	10
"S"	"S"	F.	—	13	—	—	—	—	—	—	—	—	—	—	—	—	—	—
"S"	R III	F.	—	16	—	—	—	—	—	—	—	1	2	—	1	—	4	12·2
"S"	"S"	F.	+	21	—	—	—	—	—	1	—	—	—	—	—	—	1	9
"S"	R III	F.	+	21	—	—	2	1	2	1	—	2	1	—	2	—	11	9·2
R III	R III	M.	—	16	—	—	—	—	—	—	—	—	—	—	—	—	—	—
R III	"S"	M.	—	16	—	—	—	—	—	—	—	—	—	—	—	—	—	—
R III	R III	M.	+	14	—	—	1	3	—	1	1	1	1	—	—	—	8	8·6
R III	"S"	M.	+	16	—	—	—	—	—	1	—	—	—	—	—	—	1	9
R III	R III	F.	—	16	—	1	1	2	3	1	3	1	1	—	—	—	13	8·5
R III	"S"	F.	—	19	—	—	—	—	—	—	1	—	—	—	—	—	1	7
R III	R III	F.	+	13	—	1	2	2	—	2	1	1	—	—	—	—	9	7·7
R III	"S"	F.	+	17	—	—	—	1	—	—	—	—	—	—	—	—	1	7

and litter mates of strain " S," half of which were nursed by their own mothers and half were fostered by mothers of R III strain.

At weaning time the mice of each litter were separated according to sex, marked individually and divided as equally as possible into two groups. To one group oestrone was administered; the other group served as controls. Mice in the second group were allowed to breed and to bring up their litters in a normal way.

Mice of both sexes were painted twice weekly with oestrone dissolved in chloroform. The painting of mice was continued for twelve months.

The mice were kept under similar conditions and on a diet of rat-cake cubes with an unlimited supply of water.

The results of the administration of oestrone to males and females of the " S " low cancer strain and R III high cancer strain, and to mice of both sexes belonging to the " S " strain foster nursed by R III strain females and to the R III strain fostered by " S " strain mothers, are presented in Table I. Mice which died before the appearance of the earliest tumour are not included.

The appearance of tumours in particular months after administration of oestrone is shown in Table II.

Altogether 72 litters were employed, 36 litters of strain " S " comprising 160 mice, and 36 litters of RIII strain comprising 137 mice.

The parts played by the milk factor, susceptibility and oestrogenic hormones in the origin of spontaneous and induced mammary cancer are shown in Tables III and IV.

The tables represent the findings more clearly than any description in words, but it is justifiable to point out that the artificial administration of oestrone in female mice of the R III strain does not increase, but rather tends to decrease the percentage of mammary cancer. This finding confirms Lacassagne's observations.

TABLE III.

Strain of mice (females).	Suckled by—	Susceptibility.	Milk factor.	Painting with oestrone.	Number of mice.	Number of tumours.	Percentage.	Average tumour age (months).
" S "	" S "	(small)	—	—	13	0	—	—
" S "	R III	,,	+	—	16	4	25%	12·2
" S "	" S "	,,	—	+	21	1	4·7%	9
" S "	R III	,,	+	+	21	11	52·3%	9·2
R III	R III	+	+	—	16	13	81·2%	8·5
R III	" S "	+	—	—	19	1	5·2%	7
R III	R III	+	+	+	13	9	69·6%	7·7
R III	" S "	+	—	+	17	1	5·8%	7

TABLE IV.

Strain of mice (males).	Suckled by—	Susceptibility.	Milk factor.	Painting with oestrone.	Number of mice.	Number of tumours.	Percentage.	Average tumour age (months).
" S "	" S "	(small)	—	—	14	0	—	—
" S "	R III	,,	+	—	14	0	—	—
" S "	" S "	,,	—	+	19	1	5·2%	8
" S "	R III	,,	+	+	31	12	38·7%	10
R III	R III	+	+	—	16	0	—	—
R III	" S "	+	—	—	16	0	—	—
R III	R III	+	+	+	14	8	57·1%	8·6
R III	" S "	+	—	+	16	1	6·2%	9

DISCUSSION.

The present results confirm and extend the results of Shimkin and Andervont (1941), Shimkin and Grady (1941), Twombly (1940) and Bittner (1940, 1941), and show clearly the part played by the milk factor present in the milk of high cancer strain females in the aetiology of mammary carcinoma, whether of spontaneous origin or induced by oestrogenic hormones. This can be seen in low cancer strain females foster nursed by high cancer strain mothers or in high cancer strain females fostered by low cancer strain females, untreated and after treatment with oestrogenic hormones. The presence of the milk influence increases the susceptibility of mice to the induction of breast cancer after administration of oestrone. This can also be seen in low cancer strain males fostered by high cancer strain females, as compared with low cancer strain males nursed by their own mothers and treated with oestrone. The lack of the active milk influence decreases the susceptibility of mice to the induction of breast cancer irrespective of their genetic constitution. This can be seen also in high cancer strain females and males fostered by low cancer strain mothers and treated with oestrone as compared with their litter mates nursed by their own mothers and treated with oestrone. The consequence of the absence of milk factor can be seen in low cancer strain males and females nursed by their own mothers and treated with oestrone, as compared with their litter mates fostered by high cancer strain mothers and painted with oestrone.

It can be stated in confirmation of the findings previously reported that the administration of oestrogenic hormones induces mammary cancer in mice with an inherited high degree of susceptibility and with an active milk influence, and in mice with an active milk influence but with a low degree of susceptibility. Similarly spontaneous breast cancer develops in mice susceptible to it and having an active milk influence, or in mice which although less susceptible have an active milk influence.

Few tumours develop after treatment with oestrone if the milk influence is absent or present in small amount. The tumours which developed in a few cases in high cancer strain mice fostered by low cancer strain mothers appeared in mice of one litter which was kept for longer than 24 hours with their mother, and therefore obtained a certain amount of active milk influence sufficient to produce tumours in some of the mice.

Thus the results obtained in the present experiments show that the milk of high cancer strain females contains a factor of great importance not only in the development of spontaneous breast cancer, but also in the production of breast cancer by oestrogenic hormones.

SUMMARY.

The influence of milk factor on the development of breast cancer in "S" low cancer strain and R III high cancer strain mice nursed by their own mothers, and in their litter mates foster nursed by high and low cancer strain mothers after administration of oestrogenic hormones has been investigated.

The results show that the milk influence contained in the milk of high cancer strain females has an important influence on the development of mammary cancer, whether of spontaneous origin or induced by oestrogenic hormones.

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TRANSMISSION OF THE MAMMARY TUMOUR INCITING FACTOR BY SPLENIC GRAFTING.

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BITTNER (1939) showed that the mammary tumour inciting factor which is normally conveyed by the milk of high cancer strain females can also be transmitted by grafts of lactating mammary tissue, of spleen and thymus of high cancer strain females.

Woolley, Law and Little (1941) showed that this factor is also present in the blood of high cancer strain mice.

Andervont, Shimkin and Bryan (1942) also found that the mammary tumour inciting factor can be transmitted by lactating mammary tissue and spleen of high cancer strain females.

The present experiments were undertaken to ascertain whether the active agent is present in the normal spleen of low cancer strain mice foster nursed by high cancer strain females in a concentration sufficient to produce mammary tumours in mice susceptible to the development of breast cancer.

EXPERIMENTAL PART.

Mice of R III high cancer strain, " S " low cancer strain and S × R III low cancer strain hybrids, born and raised in the Laboratories of the Imperial Cancer Research Fund, were used in the experiments.

The incidence of mammary cancer in R III high cancer strain females, as reported in a previous paper (Dmochowski and Gye, 1943), is about 83 per cent. at an average age of 8·5 months, while the incidence of mammary cancer in " S " strain females is less than 1 per cent.

" S " strain females when foster nursed by R III high cancer strain mothers have, as previously reported, an incidence of mammary tumours amounting to 29·6 per cent. at an average age of 15 months.

S × R III hybrids were employed in the present experiments as test mice, as in our experience these hybrids are susceptible to the development of mammary cancer. R III females when mated to " S " strain males give hybrid females with an incidence of about 80 per cent. at an average age of 9·4 months. No spontaneous mammary cancer has occurred as yet in hybrid females obtained by mating " S " strain females to R III strain males.

The experiment was started in October, 1941, by brother to sister matings of " S " strain mice. Pregnant " S " strain females were examined twice daily, and as soon as the young were born they were transferred to R III strain foster mothers. At weaning time the " S " strain females fostered by R III strain mothers were killed under anaesthesia and their spleens removed under aseptic conditions. Each spleen was cut into four equal portions and each portion was injected subcutaneously into S × R III hybrid mice, 4 weeks of age.

The S × R III hybrid mice of each litter were marked individually, separated according to sex and divided as equally as possible into three groups.

One group comprised mice for administration of splenic tissue ; males of this group were painted with oestrone for a period of 12 months; females were forcibly bred by removing each litter 24 hours after birth. In the second group were S × R III mice which were not inoculated with spleen ; the males of this group were painted twice weekly with oestrone and the females were forcibly bred. Mice of the third group were allowed to breed and to bring up litters in a normal way.

All mice were kept under similar conditions and on a diet of " Rat-cake " cubes with an unlimited supply of tap-water.

The results of the experiment are summarized in Table I, in which mice are presented according to the three groups.

No tumours developed in the litter mate controls, which were allowed to breed in a normal way. Few mammary tumours developed in S × R III males painted with oestrone and in S × R III females which were forcibly bred.

This result confirms the previous findings that only few or no tumours develop in susceptible mice after treatment with oestrone if the mammary tumour inducing factor is lacking (Dmochowski and Gye, 1944).

TABLE I.

Sex.	Experimental mice. Splenic grafts. Hormonal factor.	Hormonal factor.	Controls.
S × R III males	53·8 per cent.	5·5 per cent.	0 per cent.
Painted with oestrone	7 out of 13 mice 10·4 months	1 out of 18 mice 12 months	0 out of 19 mice 17 months
S × R III females	57·1 per cent.	6·2 per cent.	0 per cent.
Forcibly bred	8 out of 14 mice 10 months	1 out of 16 mice 14·5 months	0 out of 21 mice 19 months

The subcutaneous injection of splenic tissue from " S " low cancer strain females fostered by R III high cancer strain mothers induced mammary cancer in both males and females of the S × R III hybrid strain.

It has to be pointed out that in the present experiments spleens of normal " S " strain females nursed by their own mothers were not employed for controls.

SUMMARY.

The results obtained in the experiments show that the mammary tumour inducing factor is present in the spleen of " S " low cancer strain mice foster nursed by high cancer strain R III females in a sufficient concentration to produce mammary cancer in susceptible mice.

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STABILITY OF FIBRINOGEN IN NORMAL AND PATHOLOGICAL PLASMA.

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IT is known that beef plasma fibrinogen possesses greater stability than horse plasma fibrinogen. A higher concentration of salt is thus necessary for its precipitation. Woehlisch (1929) found the difference to depend on the fibrinogen itself rather than on differences in medium. With human and rabbit plasma we observed that even plasma fibrinogen of the same species may vary in stability depending on physiological and pathological conditions.

In fluorided normal plasma stored on ice, a precipitate of fibrinogen appears after several hours. At room temperature or at 37° C. no precipitate forms at the same time. The precipitation is reversible, since the fresh precipitate dissolves easily when warmed to 37° C. The precipitated substance thus belongs to the cold-sensitive protein group (Stein and Wertheimer, 1942). It was found to be identical with a plasma fibrinogen fraction precipitated by 20 per cent. saturation with ammonium sulphate.

The cold precipitate is diminished or absent in cases of severe liver diseases, but is abundant in haemorrhage, prolonged hunger and in pregnancy. The amount of the labile fibrinogen fraction of plasma varies in the same way.

In the following, properties of the cold-precipitable plasma fraction and data showing its dependence on physiological and pathological conditions are reported.

METHODS.

The *cold-sensitive plasma fraction* was prepared by cooling fluorided plasma (1·5 per cent. NaF) for 24 hours at 4°–7° C. The precipitate is centrifuged and washed with ice cold saline. For quantitative estimation the washed precipitate is digested in a sulphuric acid mixture according to Hawk and Bergeim (1938) and the protein content is determined by direct nesslerization.

The *labile fibrinogen fraction* was prepared by adding one volume of saturated ammonium sulphate solution to four volumes of oxalated plasma. The precipitate was washed with 20 per cent. saturated ammonium sulphate solution, dissolved in saline and dialyzed against 0·9 per cent. sodium chloride.

For quantitative estimation the labile fraction is precipitated from oxalated plasma by the addition of 0·8 volumes of saturated sodium chloride solution. The precipitate is washed with 44 per cent. saturated sodium chloride solution and the protein content is determined as above.

The *stable fibrinogen fraction* is prepared by adding 1/10 volume of saturated ammonium sulphate solution to the filtrate obtained after the precipitation of the

labile fraction. The precipitate formed is washed with 27 per cent. saturated ammonium sulphate. The precipitate is dissolved in saline and dialyzed, as is the labile fraction.

Total fibrinogen was determined by precipitating it from oxalated plasma with $CaCl_2$ and subsequent estimation of the protein in the fibrin clot as described above.

RESULTS.

Precipitation in the Cold.

When normal fluorided plasma is stored at 4° C. a precipitate begins to appear after 2–4 hours. On further cooling the precipitate becomes more voluminous until sometimes a gelatinous mass is formed. Fresh precipitates redissolve on warming to 37° C. After standing for 4–5 days, however, the precipitate becomes completely insoluble. When fluorided plasma is kept at 20° C. only a slight precipitate appears, and at 37° C. the plasma remains clear.

When other anticoagulants, such as oxalate, citrate, heparin or hirudin, are used instead of fluoride, precipitation in the cold begins only after 2–3 days, or in some plasmata does not appear at all before the onset of irreversible fibrin formation. If sodium fluoride is added to such plasmata, cold precipitation takes place as in fluorided plasma. When concentrations of oxalate many times higher than those generally used for the prevention of blood-clotting are employed, cold precipitation appears regularly in several hours. Sodium-chloride, bromide, iodide and sulphate added to hirudin plasma in concentrations equimolar to those of sodium fluoride used (1·5 per cent.) do not cause cold precipitation but tend rather to stabilize the solution.

Identity of the Cold Precipitate.

The following considerations indicate that the cold precipitate is a fibrinogen fraction: (1) It is present only in plasma and not in serum. (2) After cold precipitation the supernatant liquid shows progressive diminution in fibrinogen content to zero. (3) When redissolved in warm saline solution the precipitate clots upon addition of thrombin (fresh serum). (4) The cold precipitate may be salted out of solution by 20 per cent. saturation with ammonium sulphate, 33 per cent. saturation with sodium sulphate and 44 per cent. saturation with sodium chloride.

Such low salt concentrations precipitate only the most labile fraction of plasma fibrinogen. Thus it may be concluded that the cold precipitate is identical with a labile fibrinogen fraction. This conclusion is supported by the fact that the quantity of fibrinogen precipitated in the cold is dependent upon the concentration of labile fibrinogen in the plasma.

Iso-electric Point.

The pH of maximum flocculation was determined in McIlvaine's buffer solutions. Mixtures were made up of 2 ml. buffer solution, 1 ml. protein solution in 0·9 per cent. sodium chloride, and 0·5 ml. alcohol 96 per cent. With this method, maximum flocculation of the cold precipitate occurred at pH 4·4. This value is identical with that found, using the same method for both labile and stable fibrinogen. This is in accordance with the findings of other authors that fibrinogen when subjected to cataphoresis yields only one fraction.

Conversion of Stable into Labile Fibrinogen.

When fluorided plasma is kept in the cold for more than a week, all the plasma fibrinogen, labile as well as stable, precipitates. This suggests that stable fibrinogen

TABLE I.—*Labilization of Fibrinogen.*

Plasma preparations are stored at 4° C. The resulting cold precipitate is removed daily and the plasma returned to the ice box.

Plasma preparation.	Days in cold.	Cold precipitate, mg./100 ml.	Labile fibrinogen, mg./100 ml.	Cold precipitate + labile fibrinogen, mg./100 ml.
Oxalated plasma + 1 per cent. NaF. (Initial total fibrinogen 0·27 per cent.)	0	—	98	98
	1	26	133	159
	2	49	116	165
	4	84	90	174
	6	98	166	264
The same as above but $Mg(OH)_2$ treated. (Initial fibrinogen 0·25 per cent.)	0	—	98	98
	1	18	96	114
	2	30	88	118
	4	37	66	103
	6	41	112	153
$Mg(OH)_2$ treated (Initial fibrinogen 0·21 per cent.)	0	—	56	56
	1	16	42	58
	3	18	40	58
Same plasma + eluate from Mg $(OH)_2$ adsorbate	0	—	56	56
	1	24	70	94
	3	41	64	105

is gradually converted into the labile form. It could be shown that fibrin formation is not involved in this precipitation, since when the precipitate is removed daily it redissolves on warming to 37° C.

No such labilization occurred in oxalated plasma or in plasma adsorbed with Mg $(OH)_2$ according to Smith, Warner and Brinkhous (1934), even when sodium fluoride was added after the adsorption. Table I shows that whilst in fluorided complete plasma all the fibrinogen present is gradually converted into cold precipitate and dissolved labile fibrinogen, in $Mg(OH)_2$-adsorbed plasma the sum of the cold precipitate and labile fibrinogen does not markedly exceed the initial labile fibrinogen content. Thus, in magnesia-treated plasma only preformed labile fibrinogen is precipitated in the cold. Experiments to confirm this conclusion at 37° C. without the complication of cold precipitation failed to achieve the desired result, since plasma in these conditions clots within several hours.

$Mg(OH)_2$ removes prothrombin from the plasma. A prothrombin containing eluate was obtained by the method of Smith *et al.* (1934). Addition of this eluate to the treated plasma partly restored the ability to labilize fibrinogen. This finding is not, however, a proof that prothrombin causes the labilization of fibrinogen, since other factors than prothrombin are also removed by the adsorption and are present in the eluate. It seems unlikely that prothrombin is the only factor concerned, since no fibrinogen labilization occurred and no cold precipitation appeared in beef plasma which is comparatively rich in prothrombin. On the other hand, in man cold precipitation is correlated with the prothrombin content of the plasma, being absent in cirrhosis hepatis and abundant in haemorrhage. It is thus likely that prothrombin is concerned in the labilization, but is not the sole factor involved.

Physiological Variations in Cold Precipitation.

The extent of cold precipitation is influenced by the following: (*a*) Anaemia induced by bleeding, (*b*) starvation for 5–6 days, (*c*) liver injury by carbon-tetrachloride injections. An example of experimental anaemia is presented in Table II.

It is evident that the cold precipitate and the labile fibrinogen fraction increase with progressing anaemia (as measured by haemoglobin content). The variation in the total fibrinogen content is irregular.

Starving for 5–6 days and carbon-tetrachloride administration to rabbits also caused a stimulation of cold precipitation, always correlated with a rise in labile fibrinogen. After carbon-tetrachloride treatment, it should be noted, only fatty livers and not liver cirrhosis were obtained. As will be seen, marked reduction in cold precipitation occurred in cases of liver cirrhosis.

TABLE II.—*Labile Fibrinogen in Experimental Anaemia.*

A rabbit weighting 1 kg. was bled by heart puncture and the same amount of Locke solution was immediately injected intravenously.

Date.	Ml. blood removed.	Haemoglobin, g./100 ml.	Cold precipitate, per cent.	Labile fibrinogen, per cent.	Total fibrinogen, per cent.
11/7	20	13·0	0·04	0·12	0·48
12/7	16	8·3	0·12	0·20	0·35
13/7	8	4·8	0·18	0·26	0·36
14/7	—	5·5	0·16	0·24	0·37
16/7	—	6·3	0·07	0·15	0·34

Pathological Variations in Cold Precipitation.

Cold precipitation and labile fibrinogen were determined in a series of plasma samples from various patients in order to ascertain whether these fractions were of diagnostic value. In normal human plasma cold precipitation started regularly after 2–4 hours' cooling. The labile fibrinogen content ranged from 0·12–0·27 per cent. In

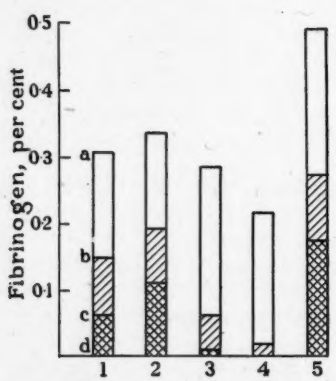

FIG. 1.—Stable, labile and cold-precipitable fibrinogen in normal and other plasma. Column 1, normal; 2, hepatitis infect.; 3, cirrhosis hepatis; 4, cirrhosis hepatis (severe); 5, late pregnancy. *a–b*, stable fraction; *b–d*, labile fraction; *c–d*, fraction precipitated in the cold after 24 hours.

cases of cirrhosis hepatis no cold precipitation was found even after 24 hours, and the labile fibrinogen content lay between 0·0–0·08 per cent. In icterus infectiosus the cold precipitate and labile fraction were increased. During pregnancy the labile fraction is especially abundant. In the last days of pregnancy, cold precipitation was often observed after 30 minutes' cooling and the labile fraction reached a concentration of 0·8 per cent. In these cases total fibrinogen content too was elevated. Enhanced cold precipitation was observed also in rheumatic fever and in anaemia haemorrhagica. Some typical examples are demonstrated in Fig. 1. Possible diagnostic values of these observations are under study.

CONCLUSIONS AND SUMMARY.

Normal plasma contains a cold-sensitive fibrinogen fraction that precipitates on cooling to 4° C. and redissolves when warmed to 37° C. Precipitation is accelerated by the presence of sodium fluoride 0·5–1·5 per cent.

The cold-sensitive fibrinogen fraction is identical with the labile fibrinogen fraction precipitated by relatively low salt concentrations—20 per cent. saturation with ammonium sulphate, 44 per cent. saturation with sodium chloride.

The stable fibrinogen fraction in fluorided plasma is converted *in vitro* into labile cold precipitable fibrinogen. Factors involved in this conversion are examined.

In vivo the quantity of cold-precipitable fraction is increased when fibrinogen is regenerated in the body, as in cases of haemorrhage. On the other hand, when the liver, the seat of fibrinogen regeneration, is impaired, as in cirrhosis hepatis, the cold-precipitable fraction disappears.

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THE EFFECTS OF PROLONGED ADMINISTRATION OF SMALL DOSES OF ALLOXAN UPON THE ISLET TISSUE OF THE RAT PANCREAS.

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HUGHES, Ware and Young (1944), following up the work of Shaw Dunn *et al.* (1943) on the effects of the administration of a single dose of alloxan to the rat, were able to show that, in this animal, (*a*) while changes occurred in the β cells of all the islets, the most marked and the earliest changes were found in the β cells of the large islets; (*b*) the first signs of damage appeared very quickly after the administration of the drug. They suggested that all the observed changes, chemical and cytological, could be explained by assuming that alloxan had a specific destructive action on the β cells.

Further work which has been carried out on animals treated with amounts of the drug insufficient to produce signs and symptoms of diabetes has thrown fresh light on the nature of the changes produced in the islets. The results obtained, and a tentative interpretation, are summarized below.

METHODS.

The material consisted of 15 female rats. They were separated into groups of three. The animals in Group I served as controls, and received a daily injection of physiological saline. Group 2 animals were treated daily with 125 mg. of alloxan/kilo, administered subcutaneously. Group 3 animals received 25 mg./kilo, Group 4 animals 5 mg./kilo, and Group 5 animals 1 mg./kilo. One animal from each group was killed at the end of 14 days, a second after one month, and the remaining animal after two months.

The pancreas was removed immediately after death, fixed in Bouin's or Helly's fluid, embedded in paraffin, cut at 4μ and stained by Gomori's method (1941). None of the animals developed glycosuria or ketonuria during the course of the experiment.

RESULTS.

After 14 days of treatment all the large islets (for the purposes of this work islets of mean diameter 150μ and upwards have been termed large, and islets of mean diameter 50μ and below have been termed small) in the pancreas of an animal receiving daily a 125 mg./kilo dose of alloxan show degenerative changes. Some contain β cells in various stages of degranulation. In others shrunken, distorted and necrotic β cells occur. A few large islets consist entirely of peculiar agranular cells. The small islets are all undamaged.

If treatment is continued for a month some large islets presenting degenerative changes can still be found but the majority of large islets appear healthy. These

latter, however, seem to contain β cells which are much larger than normal. The small islets again escape damage.

After two months of treatment large islets cannot be found in the pancreas. The small islets are very few in number and all show evidence of damage to the β cells (e.g. degranulation and pyknosis).

With the smaller doses in the range employed the islets show little sign of damage until the administration has been continued for long periods. An animal treated daily with 25 mg. alloxan/kilo for two months shows peculiarities in the distribution of the specific granules of the β cells. These granules are gathered into a number of clumps scattered irregularly throughout the cytoplasm. Between the clumps no granules are visible. This is in striking contrast to the even distribution which can be seen in the normal cell. Some islets present cloudy swelling. When still smaller doses are employed the islets appear normal on examination by routine methods. It will be shown later, however, that such islets are by no means normal.

In view of the apparent change in β cell size which had been noted in some of the experimental animals, measurements were made of the mean β cell diameter in both large and small islets of treated and control animals. For this purpose the islets were divided into four categories :

(*a*) Islets of diameter 50μ and below.
(*b*) ,, ,, 50–75μ
(*c*) ,, ,, 75–150μ.
(*d*) ,, ,, 150μ and above.

In each animal 400 typical β cells were measured, 100 from each category. The mean

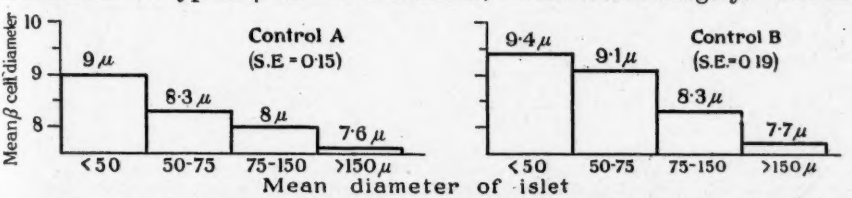

FIG. 1.—Diagram to show the inverse relationship between the mean diameter of β cells and the mean diameter of the islet in the normal rat. S.E. = standard error of difference between mean for islets below 50μ and islets above 150μ in diameter.

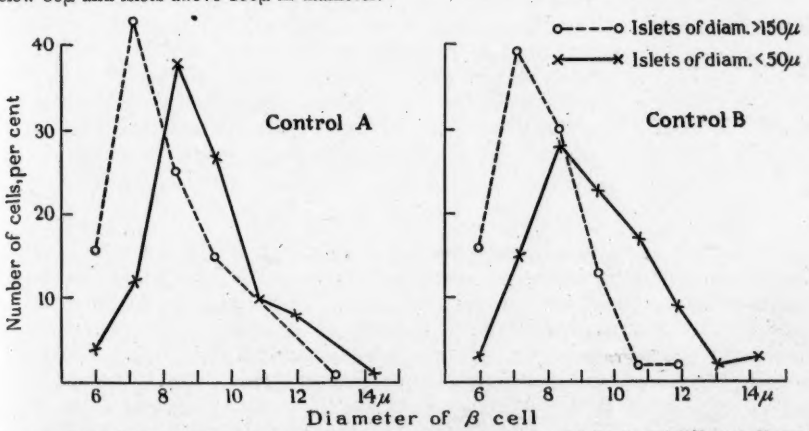

FIG. 2.—β cell distribution curve, showing the percentage incidence of β cells of different diameters in islets below 50μ and above 150μ mean diameter in the normal rat pancreas.

β cell diameter was then calculated for each size of islet. In several control animals the results were found to be agreeably consistent. The relationship between β cell size and size of islet in normal rat pancreas is shown in Fig. 1. It is clear that the mean β cell diameter decreases steadily with increase in the diameter of the islet.

A distribution curve showing the incidence of β cells of varying size (Fig. 2) demonstrates their symmetrical distribution about a peak which in the case of the large islets lies approximately at 7μ, and in the case of small islets at 8·5μ. The largest cells in the small islets are always larger than the largest cells in the large islets.

In the case of the alloxan-treated animals some striking changes occur in the form of the graphs. Rat Fb1 (Fig. 3) shows the mean β cell diameter for each category of islet in an animal treated for one month with a comparatively large dose of the drug.

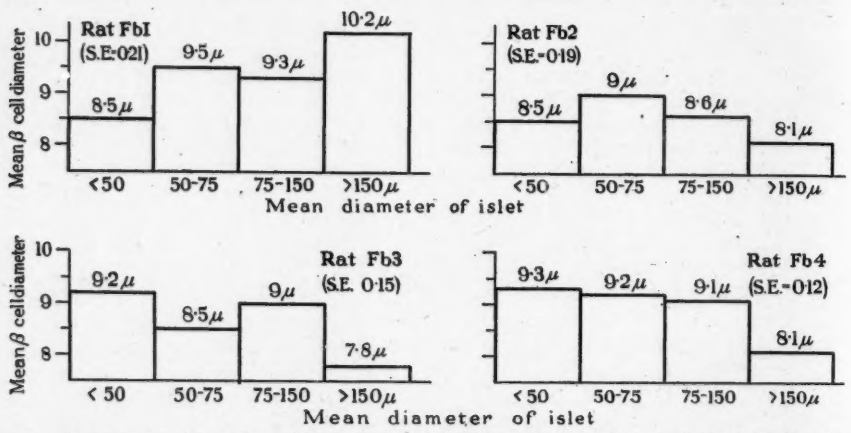

FIG. 3.—Showing the disturbance of the normal relationship between cell size and islet size after the administration of alloxan. Rat Fb1 received 125 mg. alloxan/kilo daily for 1 month; rat Fb2 received 25 mg. alloxan/kilo daily for 1 month; rat Fb3 received 5 mg. alloxan/kilo daily for 1 month; rat Fb4 received 1 mg. alloxan/kilo daily for 1 month.

There is a complete reversal in the distribution of the cells. The large islets now contain the larger cells. The distribution curves bring out the change in a very striking way. In the large islets there are two frequency peaks, at 8·5μ and 11·8μ. The largest cells in the large islets now exceed in size the largest cells in the small islets (Fig. 4). There is little change in cell size in the small islets. In the case of animals treated with smaller doses of alloxan the disturbances become less striking as the dose is reduced, until with a dose of 1 mg./kilo the normal relationships persist unchanged (Figs. 3 and 4).

DISCUSSION.

It is necessary, first, to account for the variations in β cell size which have been observed in the control animals. The mean diameter of the β cells decreases steadily with increase in the mean diameter of the islet. The difference between the mean cell diameter in small and large islets appears to be statistically significant. The standard error of this difference in the case of Control A = 0·15. The difference between the two means = 1·4μ, which is approximately ten times the standard error. In the case of Control B the difference between the means, 1·7μ, is about 8·5 times the standard error 0·198 (Fig. 1). In the large islets of the rat pancreas, and only in the large islets, shrunken cells with pyknotic nuclei are found fairly frequently. They

appear to be more numerous in very large islets. These facts would seem to suggest that, in the rat, the islets undergo a process of enlargement and maturation succeeded by a phase of involution and cell shrinkage. It has not yet been possible to determine the ultimate fate of these large islets.

The following hypothesis would seem to account satisfactorily for the changes which have been observed in the islets after the administration of alloxan. Senile β cells succumb to the toxic action of concentrations of alloxan which are too low to damage young β cells. The duration of exposure is a factor which influences the result.

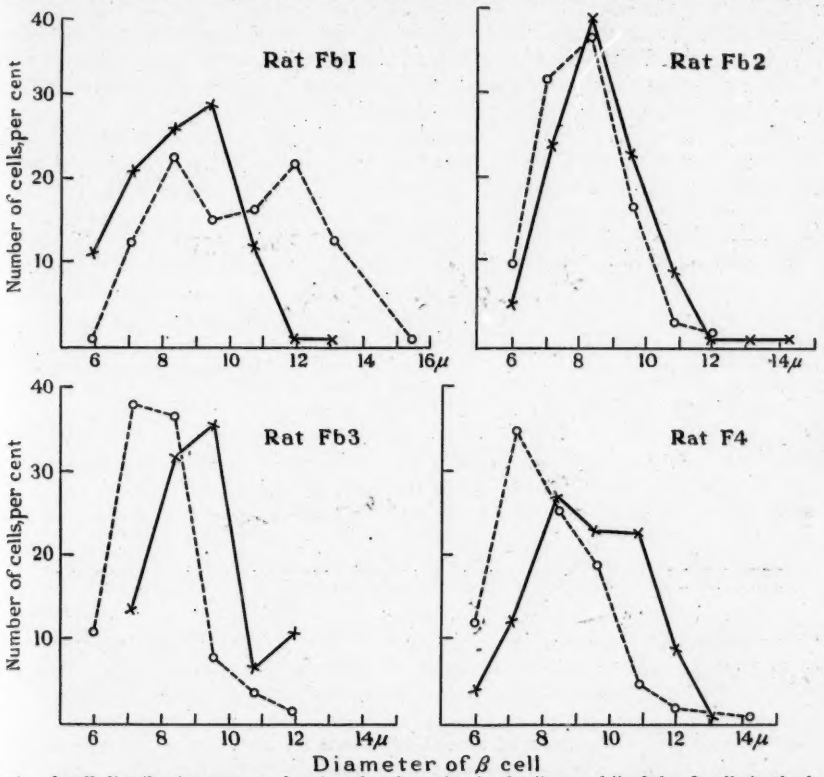

FIG. 4.— β cell distribution curves, showing the alteration in the " spread " of the β cells in the large islets after treatment with alloxan. (Dosage as indicated in Fig. 3.)

The destruction of many β cells in the large islets which is brought about in this way disturbs a physiological equilibrium and thus hastens the maturation and enlargement of replacing islets. Owing to this rapid maturation the new large islets contain young, and therefore large, β cells. Such large cells resist the action of alloxan for a considerable time. If treatment with a comparatively large dose is maintained for long periods (two months in the case of Rat Gb1) the maturing islets, becoming susceptible to the action of the drug, are destroyed continually. The ability of the pancreas to reproduce new islet tissue becomes exhausted and the total amount of islet tissue steadily declines. The animal must then inevitably become diabetic. It seems likely that this was on the point of occurring in animal Gb1 at the time of death. It must be

assumed that the senile islets which succumb to the action of alloxan are functionally active since they are replaced so rapidly by the enlargement of young islets. The increase in cell size in large islets beyond the maximum observed in the small islets of control animals may be the outcome of an increased functional load thrown upon the surviving β cells as the result of the destruction of so much islet tissue.

SUMMARY.

1. In the laboratory rat the mean diameter of the β cell is inversely related to the mean diameter of the islet.

2. Shrunken β cells with pyknotic nuclei can be found in nearly all large islets.

3. It is suggested that these changes in the β cells are the expression of a cycle, in the islets, of maturation and enlargement, followed by involution and cell shrinkage.

4. Administration of an appropriate dose of alloxan will lead to the destruction of the older β cells only. The accelerated maturation of the islets which ensues leads to disturbances in that relationship between β cell size and islet size which has been shown to hold good in the normal rat.

We are indebted to Professor F. G. Young and to Dr. L. L. Ware for the provision of the material on which this work has been carried out, and for much helpful advice.

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INFLUENZA VIRUS AS A LABORATORY CONTAMINANT.

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Bacteriologists are accustomed to the idea that occasionally unwanted organisms may find their way into culture-media and there multiply. It is less generally recognized that a contaminant virus may similarly obtain access to a suitable "medium," whether a living animal, developing chick embryo or tissue-culture. Recently developed techniques render such accidents more likely to occur, for it is possible by their aid to reveal the presence of minute amounts of virus; we refer especially to the technique of "blind passage" or "passage by faith," used in the study of influenza. Mice inoculated intranasally are killed after a few days, and, even if they show no abnormalities, suspensions of their lungs are inoculated into more mice, and so on; transmissible lesions may appear only after several passages. A trace of virus accidentally introduced at any stage has every opportunity of making its presence apparent after one or two further passages.

Two of us (Andrewes and Glover, 1944) have recently described the recovery during the recent influenza-A outbreak of two virus strains which proved serologically identical with the stock laboratory strain, PR8, and, like it, of very high mouse-virulence. These features led us to suspect that the strains might be " laboratory contaminants," even though one of them appeared in a laboratory where no work on influenza virus had been carried out for the previous 17 days. We have been led by these occurrences to collect together other instances in which laboratory contamination is suspected ; they come from various institutions in which influenza research has been carried out during the last decade. We shall refer to the workers and laboratories under letters, though we do not think that the occurrence of a virus-contaminant is any more blameworthy than a similar occurrence in the field of cultivable bacteria ; the danger is, however, less generally recognized.

The W.S. Strain of Influenza A.

Many of the instances of possible contamination to be described concern the W.S. strain of virus, isolated by Smith, Andrewes and Laidlaw in 1933 ; this strain is antigenically more distinct from the vast majority of A-strains than these are from one another, and it differs also in other biological properties. Although Burnet and Clark (1942) conclude that the W.S. virus was responsible for the 1933 epidemic, it may have been merely one of several strains active during that period ; moreover, since 1933, no W.S.-like strain has been incriminated as the agent responsible for an outbreak of influenza. W.S. virus has, however, made its appearance in various laboratories in the circumstances detailed below.

Instances of Possible " Contamination."

(i) Worker A in country Z recovered an influenza A strain serologically like W.S. from an outbreak. The strain was, at the time, the only one known to have been isolated by that worker. The Hampstead laboratory had previously sent the W.S. virus to A.

(ii) Worker B in another laboratory in country Z recovered a W.S.-like strain in similar circumstances. He also had been previously supplied with W.S. virus from Hampstead.

(iii) Worker C in country X recovered a single strain from an outbreak ; this was of W.S. type. W.S. virus had previously been sent to him. None of the numerous strains recovered from the same outbreak by other workers was closely related to W.S.

(iv) Worker D in country Y obtained a strain probably identical with W.S. from normal mice. W.S. virus had previously been sent to him. Numerous attempts by one of us (W.S.) to detect influenza virus in different stocks of normal mice have been uniformly unsuccessful.

(v) Worker E in country W recovered a strain probably identical with W.S. from an influenza patient ; this is the only strain known to have been recovered by him. He had previously been supplied with W.S. virus.

(vi) Worker F in country V recovered several strains of influenza from normal persons. One of these was sent to us and found to be exactly like W.S. We had previously sent W.S. virus to F. Our own attempts to detect human carriers of influenza virus have been unsuccessful.

These instances are suggestive, but by no means conclusive. At the time of one of the " incidents," careful comparison was made of the techniques used in the laboratory concerned and those employed by ourselves and others ; no significant differences

could be observed. The suspicions we were forming were confirmed by the following occurrences in our own laboratories or in those with which we were closely associated.

(vii) Worker G recovered W.S. virus by intranasal inoculation of mice with a third subculture in minced tissue of what was ostensibly vaccinia virus. On going back over his stored material, he also recovered W.S. virus from a first subculture in the same series, but not from the rabbit testis which had been used to initiate the culture series.

(viii) A recently isolated strain of influenza virus was sent to G by another worker. After a few mouse-passages it was found to have all the properties of W.S. virus. In order to confirm its identity, G went back to the original virus sent to him, but found, after passage, that its properties, both serologically and as regards mouse-virulence, were like those of other recently isolated strains and unlike W.S.

(ix) Worker H inoculated mice intranasally with filtered and unfiltered suspensions of a rabbit's testis subsequently proved to be infected with tuberculosis. Lesions were produced by this material, both filtered and unfiltered, and further passage revealed that W.S. virus was responsible. Later the rabbit's testis, after storage in the cold in 50 per cent. glycerol for 36 days, failed to yield any influenza virus; one would have expected the W.S. virus, had it really been present in that material, to have survived such storage.

(x) Worker I inoculated mice with secretions from a sufferer from a common cold; after a few mouse-passages W.S. virus turned up.

(xi) Worker J carried out more extensive studies. He found, as have many other workers, that some human and ferret sera are toxic to mice when given intranasally, and may kill them. From the lung lesions of mice surviving such an inoculation he made serial passages, first at intervals of seven days or more, later, when transmissible lesions appeared, at shorter intervals. A transmissible agent was obtained in five separate series, and it is highly probable that this was the mouse-pneumonitis virus which has been shown by workers in the U.S.A. and Germany to be latent in many stocks of laboratory mice. (We have ourselves found it recently in British mice.) In one series, however, an agent was recovered which, by cross-immunity tests in ferrets and mice and serological comparison by Hirst's (1942) method, seemed to be identical with W.S. virus. It is impossible to say whether, and if so at what stage, it replaced a mouse-pneumonitis virus. A detailed account of these studies by one of us (F.H.) is in preparation.

At the time of occurrence of incidents (vii), (viii), (ix), (x) and (xi), W.S. virus was under study in the laboratories concerned. At the time of the next three occurrences to be recorded, the PR8, but not W.S. strain of influenza A virus was under study; in each of these instances the virus which appeared was by serological tests and high mouse-virulence just like the PR8 strain.

(xii) Worker I recovered influenza virus like PR8 from mice inoculated intranasally with material supposed to contain typhus rickettsiae.

(xiii) Worker K inoculated into the allantoic cavity of developing eggs, ferret material containing recently isolated influenza virus not adapted to mice. The allantoic fluid of one chick embryo of the first passage agglutinated fowl R.B.C. to high titre and killed mice with specific lesions when inoculated intranasally. This strain, which proved to behave in all respects like PR8, contrasted with other strains which, immediately after they had first infected embryos, were of quite low pathogenicity for mice. On repeating this experiment with more ferret material, no adaptation to eggs or mice was achieved. Dried PR8 virus was being used in this laboratory as an antigen in Hirst tests.

(xiv) Worker L inoculated material from a patient with atypical pneumonia into

ferrets and mice. An influenza virus of full mouse-virulence was recovered from both species, and tests revealed its identity with PR8 virus. Details of this and the preceding occurrence (xiii) have recently been published (Andrewes and Glover, 1944). The striking feature of the last occurrence was that no influenza virus had been under study in the laboratory for 17 days; thus, if we suppose that contamination with PR8 virus occurred, the virus must have been persisting in the laboratory, perhaps in dust—unless worker L or an attendant responsible for the care of the animals was carrying the virus in his respiratory tract.

Infectivity of Influenza Virus in Mice.

Before discussing the significance of the above occurrences, we must deal with one potential source of "contamination," the possible transference of influenza infection from mouse to mouse within a cage or from cage to cage. Eaton (1940) has studied the conditions under which influenza can spread amongst mice within a cage. He found that it could spread readily from mice infected with heavy doses of virus, particularly if contact were over several days. But lightly infected mice were much less infectious, and contact-infection from mouse to mouse, in series, was not possible. W.S. virus would spread rather more readily than other strains. There was "no direct evidence that virus may be transmitted from infected mice in one jar to normal mice in an adjacent jar."

This experience was rather different from that at Hampstead; so in 1940 we obtained through his kindness the strain of W.S. virus he used and attempted to duplicate his experiments, using also Swiss mice, which were used by him. Groups of six mice were inoculated with heavy doses of virus and left in contact with six uninfected animals. These latter were killed after five days, and examined for lung lesions; suspensions of their lungs were then inoculated into more mice, with which more normals were placed in contact. Alternating intranasal inoculations and passages by contact were thus made; this technique in Eaton's hands permitted indefinite serial passages with W.S. virus. With us it at first succeeded admirably in two parallel series; three contact passages were successful in one series and two in the other, specific lung-lesions being found in 16/24 exposed mice killed on the fifth day. After that the contact-infection suddenly failed and was only successful in an occasional mouse; 17/20 attempts were completely negative. A renewed attempt with another ampoule of the dried virus which Dr. Eaton had sent us yielded no better results. We have repeated these experiments after an interval of 4 years and are still only able to infect an occasional mouse, on the average about 1 in 10. Our technique differed from Eaton's in that we killed and examined contact mice after five instead of ten days; a few experiments suggest that this difference is not material. We have varied our conditions in many ways, using open cages or glass jars; shavings, sawdust and other kinds of bedding; variations in humidity; and mixture with mouse pneumonitis virus. We are finally left, however, completely at a loss to explain why Eaton in the U.S.A. (and ourselves over here for a brief period) could obtain contact infection in about 75 per cent. of exposed mice, while such infection now occurs in our laboratory only infrequently. In any event, infection of "normal" mice is unlikely to explain the "contaminations" we have described, since even in Eaton's laboratory infection did not spread from one jar to another.

DISCUSSION.

The 14 "incidents" described may have diverse explanations. Some of them, such as (vii) and (ix), may be explained by such an occurrence as inadequate sterilization

of instruments or glassware. Others, for example (i), (ii), (iii) and (v), may have been genuine isolations of a virus like W.S. ; it is unlikely that that virus is unique. Its repeated appearance under the conditions described seems, however, highly suggestive. The recovery of a virus-strain lethal to mice after one egg-passage (xiii) is paralleled by facts reported by Krueger and his collaborators (1944) ; and it may mean simply that mutations in virus-properties of unexpectedly large extent can occur. But the series as a whole convinces us that influenza virus can turn up as a laboratory contaminant under conditions not yet understood. Edward (1941) has shown that influenza virus may persist, when dried on textiles, for at least a fortnight, and survival in laboratory dust is therefore a possibility.

The ease with which the W.S. strain can be distinguished from others may account for the fact that it has been implicated as a possible contaminant in the majority of the incidents recorded. Influenza virus contaminations may well occur even more frequently than our experiences suggest, but they may fail to arouse suspicion when a less distinctive strain than W.S. is concerned.

If the potentialities of influenza virus as a cause of contamination are admitted, it follows that unusual facts reported about the virus should be examined very critically. Recovery of virus by a technique which is not commonly successful, or from persons or places where others fail to find it, should be regarded with caution if the virus recovered is of high virulence and serologically like standard laboratory strains. Reports of such findings will carry more conviction if it can be stated that the standard strains have not been recently under study in the laboratory concerned.

SUMMARY.

Fourteen incidents are described as occurring in ten different laboratories ; there is strong suspicion in each of them that influenza virus has occurred as a " contaminant," thus leading to at least temporary confusion. Emphasis is laid on the importance for workers on influenza of keeping the possibility of contamination constantly in mind.

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